COMPOSITIONS AND METHODS FOR MODULATING NEURAL SPROUTING

5   This application claims priority under 35 U.S.C.
§119(e)(1) to provisional application number 60/083,472,
filed 4/29/98.

Field of the Invention

10   The present invention is directed towards methods and
compositions for inhibiting neural sprouting in neurons that
have been subjected to botulinum toxin. Also disclosed are
methods and compositions for extending the period of time
during which treatment of nerve cells with botulinum toxin
15   is effective to prevent innervation of a cell or tissue,
such as muscle cells or tissue. Such methods and
compositions are effective in the treatment of spasms or
muscular tetanus. Disclosed as well are methods and
compositions for stimulating neural outgrowth.

20

Background of the invention

        Neurotoxins, such as those obtained from *Clostridium
botulinum* and *Clostridium tetanus*, are highly potent and
specific poisons of neural cells. These Gram positive
25   bacteria secrete two related but distinct toxins, each
comprising two disulfide-linked amino acid chains: a light
chain (L) of about 50 KDa and a heavy chain (H) of about 100
KDa, which are wholly responsible for the symptoms of these
diseases.

30   The tetanus and botulinum toxins are among the most
lethal substances known to man, having a lethal dose in
humans of between 0.1 ng and 1 ng per kilogram of body

weight. Tonello et al., *Adv. Exp. Med. & Biol.* 389:251-260 (1996). Both toxins function by inhibiting neurotransmitter release in affected neurons. The tetanus neurotoxin (TeNT) acts mainly in the central nervous system, while botulinum

5 neurotoxin (BoNT) acts at the neuromuscular junction by inhibiting acetylcholine release from the axon of the affected neuron into the synapse, resulting in a localized flaccid paralysis. The effect of intoxication on the affected neuron is long-lasting and has been thought to be

10 irreversible.

The tetanus neurotoxin (TeNT) is known to exist in one immunologically distinct type; the botulinum neurotoxins (BoNT) are known to occur in seven different immunogenic types, termed BoNT/A through BoNT/G. While all of these

15 types are produced by isolates of *C. botulinum*, two other species, *C. baratii* and *C. butyricum* also produce toxins similar to /F and /E, respectively. *See* e.g., Coffield et al., *The Site and Mechanism of Action of Botulinum Neurotoxin* in *Therapy with Botulinum Toxin* 3-13 (Jankovic J.

20 & Hallett M. eds. 1994), the disclosure of which is incorporated herein by reference.

Regardless of type, the molecular mechanism of intoxication appears to be similar. In the first step of the process, the toxin binds to the presynaptic membrane of

25 the target neuron through a specific interaction between the heavy chain and a cell surface receptor; the receptor is thought to be different for each type of botulinum toxin and for TeNT. The carboxy terminus of the heavy chain appears to be important for targeting of the toxin to the cell

30 surface.

In the second step, the toxin crosses the plasma
membrane of the poisoned cell. The toxin is first engulfed
by the cell through receptor-mediated endocytosis, and an
endosome containing the toxin is formed. The toxin then
5   escapes the endosome into the cytoplasm of the cell. This
last step is thought to be mediated by the amino terminus of
the heavy chain, which triggers a conformational change of
the toxin in response to a pH of about 5.5 or lower.
Endosomes are known to possess a proton pump which decreases
10   intra-endosomal pH. The conformational shift exposes
hydrophobic residues in the toxin, which permits the toxin
to embed itself in the endosomal membrane. The toxin then
translocates through the endosomal membrane into the
cytosol.

15   The last step of the mechanism of botulinum toxin
activity appears to involve reduction of the disulfide bond
joining the heavy and light chain. The entire toxic
activity of botulinum and tetanus toxins is contained in the
light chain of the holotoxin; the light chain is a zinc
20   (Zn^{++}) endopeptidase which selectively cleaves proteins
essential for recognition and docking of neurotransmitter-
containing vesicles with the cytoplasmic surface of the
plasma membrane, and fusion of the vesicles with the plasma
membrane. TxNT, BoNT/B BoNT/D, BoNT/F, and BoNT/G cause
25   degradation of synaptobrevin 2 (also called vesicle-
associated membrane protein (VAMP)), a synaptosomal membrane
protein. Most of the VAMP present at the cytosolic surface
of the synaptic vesicle is removed as a result of any one of
these cleavage events. Each toxin specifically cleaves a
30   different bond.

BoNT/A and /E selectively cleave the plasma membrane-associated protein SNAP-25; this protein is bound to and present on the cytosolic surface of the plasma membrane. BoNT/C cleaves syntaxin, an integral protein having most of

5   its mass exposed to the cytosol. Syntaxin interacts with the calcium channels at presynaptic terminal active zones. *See* Tonello et al., *Tetanus and Botulism Neurotoxins in Intracellular Protein Catabolism* 251-260 (Suzuki K & Bond J. eds. 1996), the disclosure of which is incorporated by

10 reference as part of this specification. BoNT/C_1 also cleaves SNAP-25 at a peptide bond next to that cleaved by BoNT/A.

Both TeNT and BoNT are taken up at the neuromuscular junction. BoNT remains within peripheral neurons, and

15 blocks release of the neurotransmitter acetylcholine from these cells. Through its receptor, TeNT enters vesicles that move in a retrograde manner along the axon to the soma, and is discharged into the intersynaptic space between motor neurons and the inhibitory neurons of the spinal cord. At

20 this point, TeNT binds receptors of the inhibitory neurons, is again internalized, and the light chain enters the cytosol to block the release of the inhibitory neurotransmitters 4-aminobutyric acid (GABA) and glycine from these cells. Id.

25 Because of its specifically localized effects, dilute preparations of BoNT have been used since 1981 as therapeutic agents in the treatment of patients having various spastic conditions, including strabismus (misalignment of the eye), bephlarospasm (involuntary eyelid

30 closure) and hemifacial spasm. *See e.g.*, Borrodic et al.,

Pharmacology and Histology Botulinum Toxin in *Therapy with Botulinum Toxin* 3-13 (Jankovic J. & Hallett M. eds. 1994), incorporated by reference herein. Of the seven toxin types,
BoNT/A is the most potent of the BoNTs, and the most well
5 characterized. Intramuscular injection of spastic tissue
with dilute preparations of BoNT/A has been also used
effectively to treat spasticity due to brain injury, spinal
cord injury, stroke, multiple sclerosis and cerebral palsy.
The extent of paralysis depends on both the dose and dose
10 volume delivered to the target site. Typically, the
neurotoxin is administered in a preparation that also
contains several non-toxic proteins as well, including
hemagglutins and associated glycoproteins that assist in
maximizing its stability and presentation to the target
15 motor neuron.

Typically there is a 24 to 72 hour delay between the
administration of the toxin and the onset of the clinical
effect. Exposure to the toxin causes denervation atrophy.
See e.g., Dutton J., *Acute and Chronic Effects of Botulinum*
20 *Toxin in the Management of Blepharospasm*, in *Therapy with*
Botulinum Toxin at 199, incorporated by reference herein.
Although the therapeutic application of BoNT has been
extraordinarily effective, what side effects have been
observed are mainly immediate effects associated with the
25 treatment event itself. Peripheral effects causing weakness
in adjacent muscles may sometimes occur; these effects
usually do not persist beyond 1 to two weeks. The specific
manifestations of these effects upon adjacent muscle groups
depend upon the particular indication being treated. For
30 example, patients treated for blepharospasm sometimes

experience ptosis, and swallowing problems may occur after injection of neck muscles for torticollis.

Other possible consequences of treatment include the potential for overdosage through miscalculation or

5   differences in activity between different preparations of the toxin, generalized fatigue, and the potential for allergic reactions.

A feature of treatment with BoNT/A, and other clostridial neurotoxin types, is that the paralytic action

10   is temporary with symptoms reappearing in patients within a few months after toxin injection. This characteristic has been thought to be associated with the observed sprouting of nascent, synaptically active processes at the neuromuscular junction (NMJ). The production of such sprouts following

15   BoNT/A therapeutic treatment has appeared to contribute to the reinervation of the treated tissue and therefore the need for repeated serial injections of the toxin.

The duration of paralytic action caused by clostridial neurotoxin and the extent of sprouting, appears to depend on

20   the neurotoxin subtype studied and therefore appears to be related to the specific SNARE target cleaved by each neurotoxin. Thus, BoNT/A and BoNT/C_1, which cleave the t-SNARE protein SNAP-25 within one amino acid of each other, cause a long lasting paralysis with a long average sprout

25   length observed. BoNT/F, which cleaves the v-SNARE protein VAMP, causes a paralysis of shorter duration and sprouts of shorter average length. BoNT/E, which cleaves SNAP-25 at a different position than that of BoNT/A and BoNT/C_1 (thereby liberating a SNAP fragment of different size from the plasma

30   membrane), has a short duration period of about 5 days, and

virtually no neural sprouting is observed. Without wishing
to be bound by theory, these observations suggest that
neural sprouting and duration of paralysis are normally
related events, and that the cleavage products of BoNT
5   proteolytic digestion (i.e., the liberated fragment or the
membrane-bound fragment) can directly or indirectly
regulate, or be coregulated with, neural sprouting.

It would therefore be advantageous to design a method
whereby the sprouting phenomenon may be uncoupled from
10   duration of therapeutic effect, and thus delayed, blocked or
attenuated so as to prolong the effects of injection of
tissue with toxin. Although the experiments described
herein utilize a BoNT/A preparation, it will be recognized
by those of skill in the art that the methods shown herein
15   will be suitable for employment using other clostridial
toxins, such as BoNT/B through /G, and TeNT, in which a
sprouting pathway can be observed.

Additionally, it would be advantageous to provide
herein compositions effective for the inhibition or
20   prevention of the sprouting phenomenon. Such compositions
and methods would lessen the need for patients to undergo
repeated neurotoxin treatment.

Summary of the Invention

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The present invention concerns methods for increasing
the period of time between therapeutic treatments of neural
tissue with a clostridial neurotoxin; thus the method
provides a method of increasing the effectiveness of such
30   treatments. A direct advantage of such methods is an

increased therapeutic "life" and a concomitant lessening in
the required frequency of treatment of the patient with
neurotoxin. Reducing frequency of treatment would provide
less opportunity for a patient to experience the side

5 effects described above that are observed following
treatment, but which tend to subside long before the
effectiveness of the toxin in the target area has subsided.
Additionally, reduced frequency of treatment provides less
opportunity for miscalculation of dosage amount and other

10 treatment-specific risks.

Accordingly, an aspect of the invention concerns a
composition comprising a first agent comprising a
clostridial neurotoxin for use as a therapeutic agent and a
second agent able to extend the duration of therapeutic

15 benefit of said first agent, wherein the second agent is
effective to attenuate the production of nerve terminal
sprouts following treatment of a neuromuscular junction with
the clostridial neurotoxin.

In a particular embodiment of this aspect of the

20 invention, the first and second agent may comprise a single
entity which is provided the patient in a single treatment
session. For example, the entity may comprise a single
molecule, or a disulfide-linked multichain polypeptide.
Additionally or alternatively, the entity may comprise one

25 or more adsorbed or linked heterogroup, such as a small
organic molecule or a nucleic acid linked thereto. The
entity preferably comprises both the receptor binding and
translocation activities of a clostridial heavy chain and an
active portion of a clostridial toxin light chain. The

30 light chain may also comprise an auxiliary enzymatic

activity, such as a ribonuclease, which specifically cleaves
a nucleic acid encoding an intraneuronal factor which is
responsible for the expression, activation and/or secretion
of neurotrophic factors or cell adhesion molecules. In a
5   preferred embodiment, such an auxiliary activity is provided
by a ribozyme. By ribozyme is meant a nucleic acid or
nucleic acid analog having a sequence-specific nuclease
activity; the construction and use of ribozymes are well
known in the art; see e.g., Cech, T., *Science* 236:1532-
10   1539(1987); Cech, T. R., *Curr. Opin. Struct. Biol.* 2:605-609
(1992); and Usman et al., *Nucleic Acids & Mol. Biol.* 10:243-
264 (1996), the disclosures of which are hereby incorporated
by reference herein. By nucleic acid analog is meant a
polymeric molecule able to form a sequence-specific hybrid
15   with a target single-stranded nucleic acid; such analogs may
contain modified nucleotides (or ribonucleotides) such as
3'-O methyl nucleotides, phosphorothioate modified
nucleotides, methylphosphonate nucleotides, or nucleotide
bases separated by a peptide-like bond.
20   Alternatively, a nucleic acid or nucleic acid analog
comprised in the single entity referred to above may be an
antisense agent able to selectively bind to a nucleic acid
encoding an intraneuronal factor which is responsible for
the expression, activation and/or secretion of neurotrophic
25   factors or cell adhesion molecules. This antisense agent
may further provide a double-stranded substrate for the
action of an intracellular RNAse H activity. Details
concerning certain embodiments of these aspects of the
invention are contained in *e.g.*, Dolly et al., International
30   Publication No. WO95/32738, entitled *Modification of*

Clostridial Toxins for Use as Transport Proteins and Uherek
et al., *J. Biol. Chem.* 273:8835-8841 (1998). These two
references are incorporated by reference as part of the
present application.

5   A nucleic acid moiety linked polypeptide portion of the
entity may encode a protein or polypeptide having the
ability to be expressed within a neuron and to directly or
indirectly regulate the expression, activation and/or
secretion of neurotrophic factors or cell adhesion
10   molecules.

         In preferred aspects of the invention, the second agent
is selected from the group consisting of agents able to
compete with, down-regulate, or neutralize the effects of:
IGFI, IGF II, a neurotrophic factor, leukemia inhibitory
15   factor, a nerve cell adhesion molecule and neural agrin. In
a more preferred aspect of the invention, the neurotrophic
factor is selected from the group consisting of: ciliary
neurotrophic factor, NT-3, NT-4, and brain-derived
neurotrophic factor and/or the nerve cell adhesion molecule
20   is selected from the group consisting of tenascin-C,
ninjurin, neural cell adhesion molecule.

         Applicants have surprisingly discovered that recovery
of neural function following poisoning of nerve terminals
with clostridial neurotoxin involves two distinct and
25   apparently coordinated events. First, the poisoned endplate
becomes synaptically inactive. Shortly thereafter the
endplate elaborates thin nascent axon neural processes.
These processes or "sprouts" are synaptically competent
after about 14 days following treatment with clostridial
30   neurotoxin. The sprouts continue growing, reaching a

maximal length and level of complexity after about 42 days
following treatment with neurotoxin. During this time, the
endplate remains synaptically inactive.

Secondly, after about 42 days, the sprouts begin to
5   regress, shortening in length and decreasing in complexity.
At the same time, the original endplate begins to become
synaptically active, undergoing synaptic vesicle turnover.
The increase in such turnover reaches that of the unpoisoned
endplate after about 91 days post-treatment, at
10   approximately the same time that the sprouting phenomenon
has completely regressed, and no sprouts can be observed.
These findings are reported in DePaiva et al., *Proc. Nat'l.
Acad. Sci.* 96:3200-3205 (March 1999), which is hereby
incorporated by reference herein.

15   These observations are diametrically opposed to the
prevailing wisdom in the art, in which it has largely been
assumed that the original endplate is permanently
inactivated upon treatment with clostridial toxin, and that
all return of synaptic activity is due to extension of
20   sprouts to compensate for the lack of a neurologically
functional endplate. However, as indicated, the old
paradigm has been shown by the present Applicants to be in
error, in that the poisoned endplate regains neurological
function over time, while the axon sprouts regress so that
25   after a given time period the nerve terminal appears
essentially as it did prior to treatment. Therefore,
Applicants have discovered that, far from being a permanent
feature, the axonal sprouts assume a temporary role in the
rehabilitation of the poisoned endplate.

30   Although not wishing to be limited by theory,

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Applicants believe that these results indicate that the two
events outlined above are temporally coordinated, in that a
blockage of the neural sprouting phenomenon would delay or
block the recovery of the functional endplate. Such

5   temporal coordination could be due to the secretion of one
or more factor by the damaged neural endplate (or the
inactive muscle fiber) that has neurotrophic effects
resulting in the formation of neural sprouts; these sprouts
may then elaborate a factor (either the same or different

10 from the first factor) which promotes continued sprouting.
This factor may be produced during neurite sprouting in
amounts sufficient for the reinervation of the neurotoxin-
damaged endplate even after treatment with clostridial
toxin. Thus, treatment of cells with an agent able to block

15 neural sprouting would also delay or otherwise attenuate the
ability of the treated endplate to experience return of
neurological function, and in fact may well block such
return altogether.

     As indicated, the signaling event indicating the

20 initiation of the neural sprouting phenomenon appears to be
mediated by a cytokine or other intercellular messenger. One
such agent, agrin, appear to be an important player, if not
the key molecule, in the formation of the neuromuscular
junction in development, and in neuromuscular regeneration.

25 *See* Ruegg M.A. and Bixby J. L., *Trends in Neurol. Sci.*
21:22-27 (1998), the disclosure of which is incorporated
herein by reference. Agrin appears to be present in a number
of isoforms, which result from alternative mRNA splicing.
Soluble agrin isolated from synaptic basal lamina extracts

30 (to which it binds following secretion) is able to induce

the aggregation of acetylcholine receptors in the
postsynaptic portions of muscle cells.   Agrin present in
motor neuron terminals (n-agrin) contains an insert,
relative to other agrin species, in a region termed the B/z
5   region; this insert is important in conferring the ability
on n-agrin to aggregate acetylcholine receptors in
postsynaptic tissue. Neural agrin is released by the motor-
nerve terminal and is believed by Applicants to induce post-
synaptic specialization and up-regulation of other factors,
10   such as muscle-diffusable factors, involved in the neural
sprouting response.

It is anticipated that methods which interfere with the
sprouting phenomenon (such as by the preventing the action
of agrin)   would delay the return of innervation to cells
15   which are controlled by neurons which have been
therapeutically poisoned (i.e., by BoNT or TeNT) or
otherwise damaged.   This is because, as indicated above, the
rate of return of neural function appears to be partly
dependent upon the presence of synaptically active sprouts.
20   Additionally, agents to be used in the inhibition of
sprouting may very well also delay or prevent the recovery
of neural activity of the endplate following poisoning.

Thus utilization of such methods would therefore be
expected to extend the effective period of treatment of
25   tissue with a clostridial toxin, by delaying the
regeneration of active neuromuscular synapses, both through
inhibition of sprouting and of recovery of the poisoned
endplate.

30   Detailed Description of the Invention

This invention is drawn to methods and compositions for increasing the therapeutic effectiveness of treatment of tissue with clostridial neurotoxin. This increase in
5   effectiveness is made possible by the surprising discovery that regeneration of neural tissue damaged by treatment with clostridial neurotoxin is a complex occurrence in which two coordinated events take place.

In the first of these events, the poisoned
10   neuromuscular endplate becomes synaptically inactive, demonstrating no exocytosis of synaptic vesicles and thus no transport of intracellular acetylcholine. In four days after treatment, the endplate begins to form neural sprouts that are shown to release and regenerate synaptic vesicles.
15   These sprouts grow in length and complexity until approximately 42 days following treatment with the neurotoxin; at this point the neural sprouts begin to regress and shorten. At ninety-one days following treatment, the neural sprouts can no longer be seen.

20   In the second event, simultaneously with the beginning of regression of the neural sprouts, the synaptically inactive endplate begins to regain the ability to release acetylcholine and begin to recycle synaptic vesicles. This ability, which begins at relatively low levels, increases
25   over the time period indicated above. At approximately 91 days following treatment with clostridial neurotoxin the endplate is histologically and synaptically indistinguishable from the condition of the endplate before treatment with clostridial neurotoxin.

30   These findings indicate that one may therapeutically

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intervene at one of the major steps of the sprouting

phenomenon to prevent or attenuate the neural sprouting as a

method of extending the effective period during which tissue

treated with the toxin remains paralyzed. In an initial

5   step, the muscle cells surrounding the neural endplate

respond, either sensing the inactive muscle or in response

to a signal from the poisoned nerve terminal, by producing

muscle-derived diffusable factors. The expression of a

number of muscle-derived signaling factors appears to be

10   upregulated by muscle inactivation; such factors include

insulin-like growth factors (IGF-1 and IGF-2). A factor such

as neural agrin is believed to be the initial signal

directing the muscle cell to produce the IGF molecules.

Reports have demonstrated that IGF I and IGF II effect

15   neurite outgrowth in cultured BoNT/A treated dorsal root

ganglia, and also are able to stimulate the initial

sprouting response in paralyzed mouse gluteus muscle. *See*

Caroni, P. and Schneider, C. *J. Neurosci.* 14:3378-3388

(1994) and Caroni, P., et al. *J. Cell Biol.* 125:893-902

20   (1994).

Thus, blocking the effects of such muscle derived

diffusable factors that positively affect neurite outgrowth

and sprouting may attenuate not only clostridial neurotoxin-

induced sprouting, but may also delay the eventual recovery

25   of neurotransmission at the poisoned nerve terminals. Such

blocking may occur through the use of antibodies specific

for the muscle derived diffusable factor in question, or

that are common to such muscle derived diffusable factors.

Alternatively, there are naturally occurring binding

30   proteins, such as the IGF binding proteins IGF-BP 4 and IGF-

BP 5, which can bind to, and therefore block, the

neurotrophic effect of such diffusable factors.

IGF-BP 4 has an amino acid sequence (from the amino

terminus) of:

5

MLPLCLVAALLLAAGPGPSLGDEAIHCPPCSEEKLARCRPPVGCEELVREPGCGCCATCA
LGLGMPCGVYTPRCGSGLRCYPPRGVEKPLHTLMHGQGVCMELAEIEAIQESLQPSDKDE
GDHPNNSFSPCSAHDRRCLQKHFAKIRDRSTSGGKMKVNGAPREDARPVPQGSCQSELHR
ALERLAASQSRTHEDLYIIPIPNCDRNGNFHPKQCHPALDGQRGKCWCVDRKTGVKLPGG
10 LEPKGELDCHQLADSFRE

(SEQ ID NO: 1)

This is encoded within the nucleotide base sequence (from 5'
15 to 3') of:

```
     GTGCCCTCCG  CCGCTCGCCC  GCGCGCCCGC  GCTCCCCGCC  TGCGCCCAGC
     GCCCCGCGCC  CGCGCCCCAG  TCCTCGGGCG  GTCATGCTGC  CCCTCTGCCT
     CGTGGCCGCC  CTGCTGCTGG  CCGCCGGGCC  CGGGCCGAGC  CTGGGCGACG
20   AAGCCATCCA  CTGCCCGCCC  TGCTCCGAGG  AGAAGCTGGC  GCGCTGCCGC
     CCCCCCGTGG  GCTGCGAGGA  GCTGGTGCGA  GAGCCGGGCT  GCGGCTGTTG
     CGCCACTTGC  GCCCTGGGCT  TGGGGATGCC  CTGCGGGGTG  TACACCCCCC
     GTTGCGGCTC  GGGCCTGCGC  TGCTACCCGC  CCCGAGGGGT  GGAGAAGCCC
     CTGCACACAC  TGATGCACGG  GCAAGGCGTG  TGCATGGAGC  TGGCGGAGAT
25   CGAGGCCATC  CAGGAAAGCC  TGCAGCCCTC  TGACAAGGAC  GAGGGTGACC
     ACCCCAACAA  CAGCTTCAGC  CCCTGTAGCG  CCCATGACCG  CAGGTGCCTG
     CAGAAGCACT  TCGCCAAAAT  TCGAGACCGG  AGCACCAGTG  GGGGCAAGAT
     GAAGGTCAAT  GGGGCGCCCC  GGGAGGATGC  CCGGCCTGTG  CCCCAGGGCT
     CCTGCCAGAG  CGAGCTGCAC  CGGGCGCTGG  AGCGGCTGGC  CGCTTCACAG
30   AGCCGCACCC  ACGAGGACCT  CTACATCATC  CCCATCCCCA  ACTGCGACCG
     CAACGGCAAC  TTCCACCCCA  AGCAGTGTCA  CCCAGCTCTG  GATGGGCAGC
     GTGGCAAGTG  CTGGTGTGTG  GACCGGAAGA  CGGGGGTGAA  GCTTCCGGGG
     GGCCTGGAGC  CAAAGGGGGA  GCTGGACTGC  CACCAGCTGG  CTGACAGCTT
     TCGAGAGTGA  GGCCTGCCAG  CAGGCCAGGG  ACTCAGCGTC  CCCTGCTACT
35   CCTGTGCTCT  GGAGGCTGCA  GAGCTGACCC  AGAGTGGAGT  CTGAGTCTGA
     GTCCTGTCTC  TGCCTGCGGC  CCAGAAGTTT  CCCTCAAATG  CGCGTGTGCA
     CGTGTGCGTG  TGCGTGCGTG  TGTGTGTGTT  TGTGAGCATG  GGTGTGCCCT
     TGGGGTAAGC  CAGAGCCTGG  GGTGTTCTCT  TTGGTGTTAC  ACAGCCCAAG
     AGGACTGAGA  CTGGCACTTA  GCCCAAGAGG  TCTGAGCCCT  GGTGTGTTTC
40   CAGATCGATC  CTGGATTCAC  TCACTCACTC  ATTCCTTCAC  TCATCCAGCC
     ACCTAAAAAC  ATTTACTGAC  CATGTACTAC  GTGCCAGCTC  TAGTTTTCAG
     CCTTGGGAGG  TTTTATTCTG  ACTTCCTCTG  ATTTTGGCAT  GTGGAGACAC
     TCCTATAAGG  AGAGTTCAAG  CCTGTGGGAG  TAGAAAAATC  TCATTCCCAG
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    AGTCAGAGGA GAAGAGACAT GTACCTTGAC CATCGTCCTT CCTCTCAAGC
    TAGCCAGAGG GTGGGAGCCT AAGGAAGCGT GGGGTAGCAG ATGGAGTAAT
    GGTCACGAGG TCCAGACCCA CTCCCAAAGC TCAGACTTGC CAGGCTCCCT
    TTCTCTTCTT CCCCAGGTCC TTCCTTTAGG TCTGGTTGTT GCACCATCTG
  5 CTTGGTTGGC TGGCAGCTGA GAGCCCTGCT GTGGGAGAGC GAAGGGGGTC
    AAAGGAAGAC TTGAAGCACA GAGGGCTAGG GAGGTGGGGT ACATTTCTCT
    GAGCAGTCAG GGTGGGAAGA AAGAATGCAA GAGTGGACTG AATGTGCCTA
    ATGGAGAAGA CCCACGTGCT AGGGGATGAG GGGCTTCCTG GGTCCTGTTC
    CCTACCCCAT TTGTGGTCAC AGCCATGAAG TCACCGGGAT GAACCTATCC
 10 TTCCAGTGGC TCGCTCCCTG TAGCTCTGCC TCCCTCTCCA TATCTCCTTC
    CCCTACACCT CCCTCCCCAC ACCTCCCTAC TCCCCTGGGC ATCTTCTGGC
    TTGACTGGAT GGAAGGAGAC TTAGGAACCT ACCAGTTGGC CATGATGTCT TTTCT
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    (SEQ ID NO: 3)

    IGFBP 5 has an amino acid sequence (from the amino
terminus) of:

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    MVLLTAVLLLLAAYAGPAQSLGSFVHCEPCDEKALSMCPPSPLGCELVKEPGCGCCMTCA
    LAEGQSCGVYTERCAQGLRCLPRQDEEKPLHALLHGRGVCLNEKSYREQVKIERDSREHE
    EPTTSEMAEEETYSPKIFRPKHTRISELKAEAVKKDRRKKLTQSKFVGGAENTAHPRIISA
    PEMRQESEQGPCRRHMEASLQELKASPRMVPRAVYLPNCDRKGFYKRKQCKPSRGRKRGI
    CWCVDKYGMKLPGMEYVDGDFQCHTFDSSNVE
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    (SEQ ID No. 2)

    and is encoded within a nucleotide base sequence (from 5' to
3') of:

```
 30 GGGGAAAAGA GCTAGGAAAG AGCTGCAAAG CAGTGTGGGC TTTTTCCCTT
    TTTTTGCTCCT TTTCATTAC CCCTCCTCCG TTTTCACCCT TCTCCGGACT
    TCGCGTAGAA CCTGCGAATT TCGAAGAGGA GGTGGCAAAG TGGGAGAAAA
    GAGGTGTTAG GGTTTGGGGT TTTTTTGTTT TTGTTTTTGT TTTTTAATTT
    CTTGATTTCA ACATTTTCTC CCACCCTCTC GGCTGCAGCC AACGCCTCTT
 35 ACCTGTTCTG CGGCGCCGCG CACCGCTGGC AGCTGAGGGT TAGAAAGCGG
    GGTGTATTTT AGATTTTAAG CAAAAATTTT AAAGATAAAT CCATTTTTCT
    CTCCCACCCC CAACGCCATC TCCACTGCAT CCGATCTCAT TATTTCGGTG
    GTTGCTTGGG GGTGAACAAT TTTGTGGCTT TTTTTCCCCT ATAATTCTGA
    CCCGCTCAGG CTTGAGGGTT TCTCCGGCCT CCGCTCACTG CGTGCACCTG
 40 GCGCTGCCCT GCTTCCCCCA ACCTGTTGCA AGGCTTTAAT TCTTGCAACT
    GGGACCTGCT CGCAGGCACC CCAGCCCTCC ACCTCTCTCT ACATTTTTGC
    AAGTGTCTGG GGGAGGGCAC CTGCTCTACC TGCCAGAAAT TTTAAAACAA
    AAACAAAAAC AAAAAAATCT CCGGGGGCCC TCTTGGCCCC TTTATCCCTG
    CACTCTCGCT CTCCTGCCCC ACCCCGAGGT AAAGGGGGCG ACTAAGAGAA
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        GATGGTGTTG CTCACCGCGG TCCTCCTGCT GCTGGCCGCC TATGCGGGGC
        CGGCCCAGAG CCTGGGCTCC TTCGTGCACT GCGAGCCCTG CGACGAGAAA
        GCCCTCTCCA TGTGCCCCCC CAGCCCCCTG GGCTGCGAGC TGGTCAAGGA
        GCCGGGCTGC GGCTGCTGCA TGACCTGCGC CCTGGCCGAG GGGCAGTCGT
  5     GCGGCGTCTA CACCGAGCGC TGCGCCCAGG GGCTGCGCTG CCTCCCCCGG
        CAGGACGAGG AGAAGCCGCT GCACGCCCTG CTGCACGGCC GCGGGGTTTG
        CCTCAACGAA AAGAGCTACC GCGAGCAAGT CAAGATCGAG AGAGACTCCC
        GTGAGCACGA GGAGCCCACC ACCTCTGAGA TGGCCGAGGA GACCTACTCC
        CCCAAGATCT TCCGGCCCAA ACACACCCGC ATCTCCGAGC TGAAGGCTGA
  10    AGCAGTGAAG AAGGACCGCA GAAAGAAGCT GACCCAGTCC AAGTTTGTCG
        GGGGAGCCGA GAACACTGCC CACCCCGGA TCATCTCTGC ACCTGAGATG
        AGACAGGAGT CTGAGCAGGG CCCCTGCCGC AGACACATGG AGGCTTCCCT
        GCAGGAGCTC AAAGCCAGCC CACGCATGGT GCCCCGTGCT GTGTACCTGC
        CCAATTGTGA CCGCAAAGGA TTCTACAAGA GAAAGCAGTG CAAACCTTCC
  15    CGTGGCCGCA AGCGTGGCAT CTGCTGGTGC GTGGACAAGT ACGGGATGAA
        GCTGCCAGGC ATGGAGTACG TTGACGGGGA CTTTCAGTGC CACACCTTCG
        ACAGCAGCAA CGTTGAGTGA TGCGTCCCCC CCCAACCTTT CCCTCACCCC
        CTCCCACCCC CAGCCCCGAC TCCAGCCAGC GCCTCCCTCC ACCCCAGGAC
        GCCACTCATT TCATCTCATT TAAGGGAAAA ATATATATCT ATCTATTTGA
  20    GGAAAAAAAA AAAAAAAAAA AA
```

(SEQ ID NO: 4)

These binding proteins can be made synthetically or

25 cloned and produced for therapeutic purposes, while a cell

line producing a desired monoclonal antibody can be

maintained for relatively large-scale antibody production.

Cloning and general antibody methodologies are commonplace

in the art; such methodologies are disclosed within Sambrook

30 et al., *Molecular Cloning: A Laboratory Manual* (2nd ed. Cold

Spring Harbor Laboratory Press 1989), the disclosure of

which is hereby incorporated by reference as part of this

disclosure.

Rather than using competitive methods, another aspect

35 of the invention involves the use of a cholinergic special

transporter to insert a gene which produces inactive

receptors for one or more factor involved in promotion of

neural sprouting. Such receptors would maintain high

specific binding constants to their ligands, but the
biological activity of the receptors would be abrogated;
such receptors could be easily be generated and screened
through the introduction of mutations in the nucleotide

5 sequence encoding the protein, and assaying the mutants for
binding strength and biological activity. Alternatively,
the neurotrophic activities of factors produced by the
neural endplate or nascent sprouts may be inhibited through
intracellular targeting and delivery of a competitive

10 inhibitor, ribozyme, transcriptional suppresser or other
agent specifically able to block or attenuate such
activities, as described above. Such intracellular
targeting is disclosed in references such as, e.g., Dolly et
al., International Patent Publication No. WO95/32738,

15 previously incorporated by reference herein.

The second point at which intervention in the sprouting
phenomenon may be made is during the stage of axonal
outgrowth and arbor development. At this stage such
outgrowth has already been initiated, but auxiliary factors

20 appear to be necessary in order to maintain axonal growth. A
number of factors are known to affect the rates of
outgrowth, and may also effect the survivability of many
types of neurons. Such factors include ciliary neurotrophic
factor (CNTF); neurotrophins, including NT-3, NT-4, and

25 brain-derived neurotrophic factor (BDNF); and leukemia
inhibitory factor (LIF). Not only are these factors
important in establishing an initial rate of axonal
outgrowth, but they appear to either directly or indirectly
stimulate their own production -- therefore blocking the

30 initial outgrowth of sprouts may be essential in preventing

further propagation through the continued expression of such
factors.

   The same methods as disclosed above can be used to
prevent one or more of the agents listed above to manifest
5   its activity. As would be expected by those of skill in the
art in light of the present disclosure, such agents, destroy
or bind to, and therefore attenuate the activity of these
factors or their block their ability to bind to their
receptors, or inactive forms of their receptors, would be
10 useful when used in conjunction with a clostridial
neurotoxin to prevent or reduce the rate of sprouting of
neurites from the poisoned or damaged endplate.

   The third stage at which the sprouting phenomenon may
be attenuated or inhibited concerns the binding of axons to
15 the extracellular matrix. Axons are guided to cellular
processes containing the appropriate neurotransmitter
receptors by binding to components of the extracellular
matrix. Such binding involves a variety of cell-borne or
matrix associated adhesion molecules. Tenascin-C is an
20 extracellular matrix component derived from Schwann cells
that appears to bind neural processes. Ninjurin is a cell
surface adhesion molecule that is up-regulated following
peripheral nerve injury and thought to be involved in exonal
guidance. *See* Araki, T., et al., *J. Biol. Chem.* **272**:21373-
25 21380 (1997), incorporated by reference herein. Similarly,
neural-cell adhesion molecule (N-CAM), is an adhesion
molecule which is thought to be involved in binding of
neural sprouts to the extracellular matrix. The same
techniques indicated above may be employed to prevent the
30 expression or inhibit the activity of these molecules when

used as part of a clostridial neurotoxin therapy.

     Thus, in one aspect the present invention is drawn to a
method for extending the effective period during which
tissue treated with clostridial toxin is paralyzed,
5   comprising: Contacting said tissue with a composition
comprising an agent able to prevent the neuroregenitive
activity of a polypeptide selected from the group consisting
of IGF-1, IGF-2, cilary neurotrophic factor, NT-3, NT-4,
brain-derived neurotrophic factor, leukemia inhibitory
10 factor, tenascin-C, ninjurin, neural cell adhesion molecule,
and neural agrin.

     In one preferred embodiment, the agent comprises a
polypeptide able to bind to IGF-1 and/or IGF-2 in a manner
that prevents an IGF molecule from binding to or activating
15 a cell surface receptor involved in the initiation of neural
sprouting. In a most preferred embodiment the polypeptide
comprises at least a portion of a amino acid sequence
selected from the group consisting of: IGFBP-4 (SEQ ID NO:
1) or IGFBP5 (SEQ ID NO: 2). Preferably said portion
20 comprises at least 10 contiguous amino acids of said
sequence; more preferably said portion comprises at least 20
contiguous nucleotides of said sequence. Most preferably,
the portion comprises an amino acid sequence selected from
the group consisting of the entire amino acid sequence of
25 IGFBP-4 or IGFBP-5.

     Treatment of cells with such a composition may be
accomplished either before or simultaneously with treatment
with clostridial toxin. Preferably, the clostridial toxin
is a botulinum toxin. Even more preferably, the botulinum
30 toxin comprises BoNT/A. In other embodiments, the

clostridial toxin is TeNT. Agent which are able to bind to
any of these factors in a manner that inhibits their
neurotrophic activity, or which bind to the receptors for
such factors, would, in light of the present application, be
5   expected to function as agents for extending the effective
period between treatments of tissue with a neurotoxin.

Another embodiment comprises a cholinergic specific
transporter joined to a gene encoding a gene which produces
an inactive receptor for one or more factor involved in
10 promotion of neural sprouting when delivered to a neural
cell in vivo. Preferably the receptors maintain high
specific binding constants to said factor(s) and the
biological activity of the receptor is reduced or absent.
In a particularly preferred embodiment the transporter
15 comprises some or all of a clostridial neurotoxin heavy
chain, although other transporters such as the diphtheria
toxin transporter may be effective in this regard as well.

In another embodiment the invention comprises a
cholonergic specific transporter that is covalently or non-
20 covalently joined to a nucleic acid which comprises a
ribozyme or antisense nucleic acid able to specifically
destroy the nucleic acids encoding neurotrophic agents or
their receptors. Said joining may be made through methods
including, but not limited to, covalent bonding or
25 electrostatic forces.

In another embodiment, the present invention is drawn
to the methods for stimulating the outgrowth of neural
sprouts from damaged neural tissue. Such methods could be
effective ways of increasing the rate at which reinnervation
30 occurs after a neural injury. These methods comprise:

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Contacting said tissue with a composition comprising a
polypeptide which comprises a neurotrophically active domain
derived from an agent selected from a group consisting of
IGF-1, IGF-2, cilary neurotrophic factor, NT-3, NT-4, brain

5 derived neurotrophic factor, leukemia inhibitory factor,
tenascin-C, ninjurin, neural cell adhesion molecule, and
neural agrin. Such damage may be a result of neurotoxin
poisoning or due to a traumatic event, including but not
limited to nerve or spinal cord crush injuries, traumatic

10 brain injuries, glaucoma-induced damage to the retina and/or
optic nerve, or surgical trauma or injury.

The following examples illustrate various embodiments
of the present invention, and are not intended to limit the
scope of the invention, which is solely defined by the

15 claims which conclude this specification.

Example 1

Blepharospasm is a medical condition characterized by

20 uncontrolled eyelid movement. In its early stages, the
condition is characterized by excessive blinking or
fluttering of the eyelids. The condition is generally a
progressive one, in which excessive blinking is replaced in
the later stages with spasms of eye closure that interfere

25 with visual function. The spasms become more frequent and
severe, and involve the preseptal, pretarsal, and
orbicularis oculi muscle. The condition often results in
functional blindness relatively quickly (in a matter of two
to three years) after the symptoms are first encountered.

A patient suffering from moderate idiopathic
blepharospasm is treated with injections of BoNT/A toxin
preparation containing non-toxic proteins and hemagglutins
in sterile saline. Alternatively, the same toxin
5   preparation without hemagglutins may be used. The
injections are generally in the volume of 100 μl; and each
injection contains 1.25 to 2.5 units of the toxin
preparation. The injections are made into the pretarsal
orbicularis oculi of the upper lid laterally and medially
10   and in the lower lid laterally and medially. Additionally,
2.5 unit injections (100 μl each) are made lateral to the
lateral canthus and into the brow medially. Total amount of
BoNT/A toxin injected is roughly 6.25 to 12.5 units per
eye. The Bo/A toxin is provided in a sterile, preservative-
15   free saline, and the same solution is used to dilute the
BoNT/A toxin if the master preparation of it is too
concentrated.

Following injection the treated muscles are
sufficiently paralyzed due to this treatment to alleviate
20   the major symptoms of blepharospasm. Some mild concomitant
weakness in the surrounding muscle tissue is observed; these
side effects are mild and tolerated well by the patient.
The effect of this treatment lasts approximately 8 weeks,
and must be repeated at the end of this time to maintain the
25   beneficial effects.

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Example 2

A patient with blepharospasm is pre-treated with BoNT/A
toxin as indicated in Example 1 with the following
5   difference. Prior to injection with BoNT/A toxin
preparation, the patient is given a ten-fold excess of
IGFBP-4, having an amino acid sequence of SEQ ID NO: 1. The
binding protein preparation is dissolved in sterile,
preservative-free saline. Each injection is in the same
10   area as the toxin injections that follow the pre-treatment;
the volume of each injection is 100 µl. The BoNT/A toxin
preparation is injected ten minutes after the injection of
the IGFBT 4 injection.

The patient's therapeutic response to the BoNT/A toxin
15   is similar to that seen in Example 1. The duration of the
benefit provided by the BoNT/A toxin treatment is extended
to 12 weeks or more, during which time no further injection
need be made.

20   Example 3

A patient with blepharospasm is pre-treated with BoNT/A
toxin as indicated in Example 1 with the following
difference. The BoNT/A toxin has been modified to have
25   joined thereto a nucleic acid comprising a ribozyme
specifically targeted to enzymatically destroy neural agrin
mRNA. No supplemental injections are made.

The patient's therapeutic response to the BoNT/A toxin
is similar to that seen in Example 1. The duration of the
30   benefit provided by the BoNT/A toxin treatment is extended

to 12 weeks or more, during which time no further injection need be made.

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<u>Example 4</u>

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A patient with blepharospasm is pre-treated with BoNT/A toxin as indicated in Example 1 with the following difference. The BoNT/A toxin has been modified to have joined thereto a nucleic acid encoding an inactive

10 neurotrophin receptor which retains the ability to bind its target neurotrophin. No supplemental injections are made.

The patient's therapeutic response to the BoNT/A toxin is similar to that seen in Example 1. The duration of the benefit provided by the BoNT/A toxin treatment is extended

15 beyond that seen with BoNT/A alone, during which time no further injection need be made.

It will be understood that, while reference is made to BoNT/A in the Examples above, any other of the species of botulinum toxins (e.g., BoNT/B through G) could be

20 substituted therefor, with appropriate adjustments possibly necessary due to differences in specific activity of the toxin. Additionally, the light chain segment could be derived from any clostridial neurotoxin (or other neurotoxin) , with the heavy chain retaining the motor

25 neuron receptor binding and exo-vescular transport activities retained from the BoNT heavy chain.

These Examples are not intended to exhaust the embodiments of the present invention, and the invention is not to be seen as limited thereby. Further embodiments will

<div align="center">

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</div>

be disclosed within the claims that conclude this

specification.

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